Biochemistry and Biophysics Reports 15 (2018) 52–56

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

# Epitope mapping of anti-mouse podoplanin monoclonal antibody PMab-1

Shinji Yamada[a], Shunsuke Itai[a], Mika K. Kaneko[a], Satoru Konnai[b], Yukinari Kato[a,c,*]

[a] Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[b] Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
[c] New Industry Creation Hatchery Center, Tohoku University, 2-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

ARTICLE INFO

*Keywords:*
Podoplanin
PDPN
PMab-1
Epitope mapping

ABSTRACT

Mouse podoplanin (mPDPN) is a type I transmembrane sialoglycoprotein, which is expressed on lymphatic endothelial cells, podocytes of the kidney, and type I alveolar cells of the lung. mPDPN is known as a platelet aggregation-inducing factor and possesses four platelet aggregation-stimulating (PLAG) domains: PLAG1, PLAG2, and PLAG3 in the N-terminus and PLAG4 in the middle of the mPDPN protein. mPDPN overexpression in cancers has been reportedly associated with hematogenous metastasis through interaction with the C-type lectin-like receptor 2 of platelets. We previously reported a rat anti-mPDPN monoclonal antibody clone PMab-1, which was developed by immunizing the PLAG2 and PLAG3 domains of mPDPN. PMab-1 is very useful in flow cytometry, western blot, and immunohistochemical analyses to detect both normal cells and cancers. However, the binding epitope of PMab-1 remains to be clarified. In the present study, flow cytometry, enzyme-linked immunosorbent assay, and immunohistochemical analyses were utilized to investigate the epitope of PMab-1. The results revealed that the critical epitope of PMab-1 is Asp39 and Met41 of mPDPN. These findings can be applied to the production of more functional anti-mPDPN monoclonal antibodies.

## 1. Introduction

Podoplanin (PDPN/T1alpha/gp38/Aggrus) is expressed in many normal tissues, such as renal podocytes, lymphatic endothelial cells of many tissues, and pulmonary type I alveolar cells [1–4]. Several anti-mouse PDPN (mPDPN) monoclonal antibodies (mAbs), such as clone 8.1.1 or clone PMab-1, have been used in many studies [5]. However, clone 8.1.1 is produced using hamsters, and clone PMab-1 is produced using rats because it is difficult to develop anti-mPDPN mAbs using mice. Recently, we developed a rat–mouse chimeric antibody, mPMab-1 of mouse $IgG_{2a}$, which was derived from a rat PMab-1 mAb [6]. Immunohistochemical analysis showed that mPMab-1 detects podocytes of the kidney, lymphatic endothelial cells of the colon, and type I alveolar cells of the lung. Importantly, mPMab-1 was shown to be more sensitive than original PMab-1.

mPDPN possesses four platelet aggregation-stimulating (PLAG) domains: PLAG1, PLAG2, and PLAG3 in the N-terminus [1] and PLAG4 in the middle of the mPDPN protein [7]. In a previous study, PMab-1 mAb was produced against the platelet aggregation-stimulating (PLAG) domain of mPDPN [5]; therefore, PMab-1 neutralizes the interaction between mPDPN and the C-type lectin-like receptor 2 [8–10]. The administration of PMab-1 was found to reduce lymphangiogenesis in corneal suture and ear-wound healing models [11]. PMab-1 also suppressed the infiltration of thioglycollate-induced macrophages at the site of wound healing. Furthermore, the administration of PMab-1 lead to a significant suppression of the rejection reaction in a corneal transplantation model, suggesting that mPDPN is a novel therapeutic target for suppressing lymphangiogenesis and inflammation.

In the present study, we determined the binding epitope of PMab-1 to mPDPN using flow cytometry, enzyme-linked immunosorbent assay (ELISA), and immunohistochemical analyses.

## 2. Materials and methods

### 2.1. Cell line

Chinese hamster ovary (CHO)-K1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The mPDPN mutation plasmids were transfected into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific Inc., Waltham, MA, USA). Transiently transfected cells were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-

*Abbreviations:* PDPN, podoplanin; PLAG, platelet aggregation-stimulating; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DAB, 3,3-diaminobenzidine tetrahydrochloride

* Corresponding author at: New Industry Creation Hatchery Center, Tohoku University, 2-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.
*E-mail address:* yukinarikato@med.tohoku.ac.jp (Y. Kato).

https://doi.org/10.1016/j.bbrep.2018.07.002
Received 23 June 2018; Received in revised form 1 July 2018; Accepted 2 July 2018

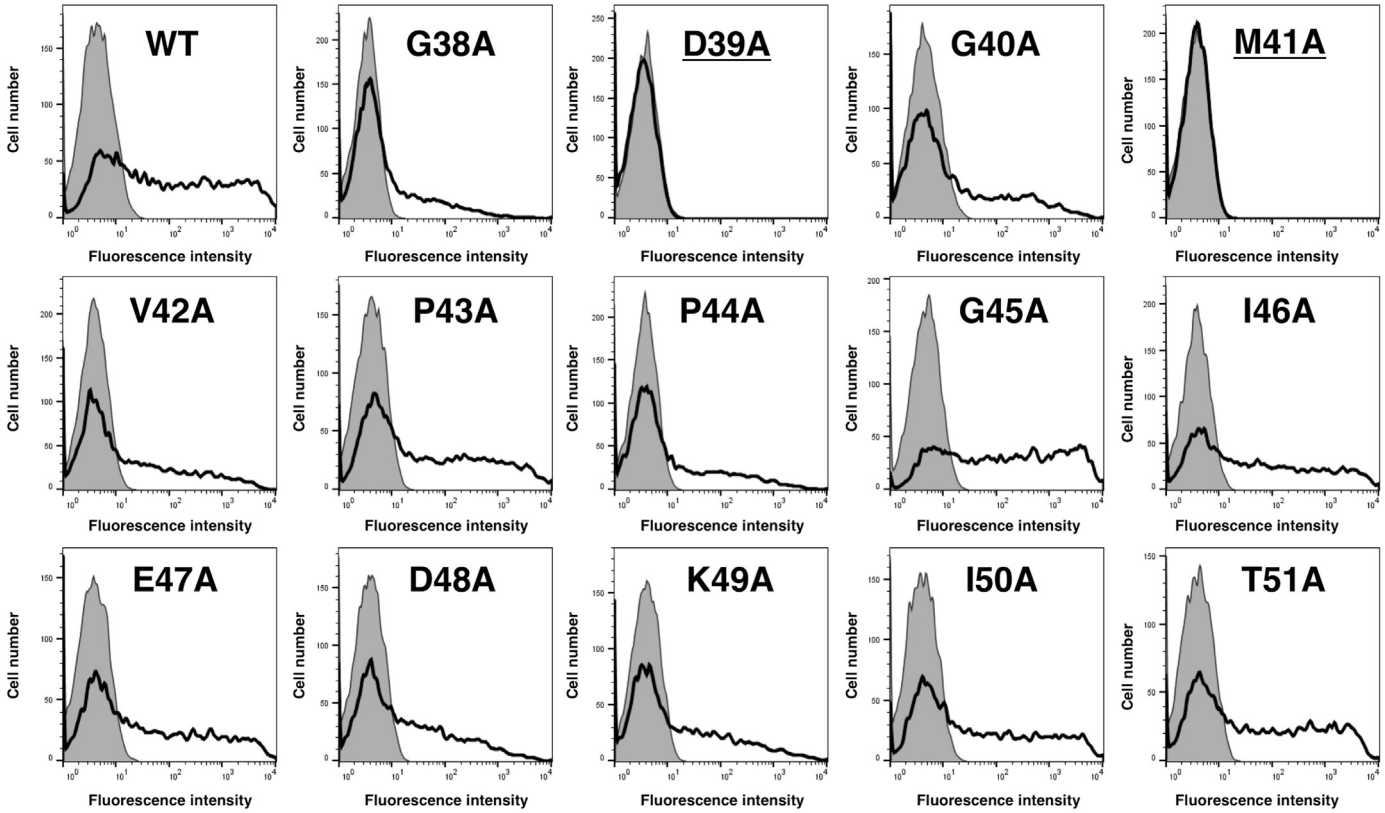


**Fig. 1. Epitope mapping of PMab-1 using point mutants of mPDPN.** Point mutants of mPDPN were analyzed using flow cytometry. Point mutants were expressed on CHO-K1 cells and then incubated with PMab-1 (2 μg/ml) or buffer control for 30 min at 4 °C, followed by treatment with corresponding secondary antibodies.

inactivated fetal bovine serum (Thermo Fisher Scientific Inc.), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 25 μg/ml of amphotericin B (Nacalai Tesque, Inc.) at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### 2.2. Production of mPDPN point mutants

The cDNA of mPDPN was subcloned into a pcDNA3 vector (Thermo Fisher Scientific Inc.) [2]. Substitutions of amino acids to alanine in the mPDPN sequence were performed using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA).

### 2.3. Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in PBS, the cells were treated with PMab-1 for 30 min at 4 °C, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were acquired using the Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

### 2.4. ELISA

Synthesized mPDPN peptides using PEPScreen (Sigma-Aldrich Corp., St. Louis, MO) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc.) at 10 μg/ml for 30 min at 37 °C. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.), the plates were incubated with purified PMab-1 (10 μg/ml), followed by a 1:2000 dilution of peroxidase-conjugated anti-rat IgG (Agilent Technologies Inc.). The enzymatic reaction was performed using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA). These reactions were performed at 37 °C with a total sample volume of 50–100 μl.

### 2.5. Immunohistochemical analyses

Histological sections (4-μm thick) of mouse tissues were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 min. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.), sections were incubated with mPMab-1 (1 μg/ml) or mPMab-1 (1 μg/ml) plus peptides (5 μg/ml) for 1 h at room temperature and treated using an Envision + kit (Agilent Technologies Inc.) for 30 min. Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies Inc.) for 2 min. Sections were counterstained with hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

## 3. Results and discussion

In a previous study, we developed a rat anti-mPDPN mAb PMab-1 by immunizing the PLAG domain of mPDPN [5]. We further produced a rat–mouse chimeric antibody, mPMab-1 of mouse $IgG_{2a}$, which was derived from a rat PMab-1 mAb [6]. Immunohistochemical analysis showed that both PMab-1 and mPMab-1 are capable of detecting podocytes of the kidney, lymphatic endothelial cells of the colon, and type I alveolar cells of the lung. Interestingly, mPMab-1 was shown to be more sensitive than original PMab-1 [6] probably because a high-sensitivity immunohistochemical kit can be used for mouse IgG. In the present study, we produced point mutants of mPDPN (proteins and synthesized peptides) and investigated the critical epitope of PMab-1 for mPDPN detection.

Because PMab-1 was developed by immunizing rats with amino acids 38–51 of mPDPN, we produced a series of point mutants of